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NOTES

Conversion of Indole to Oxindole under Methanogenic Conditions†

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When indole was incubated under methanogenic conditions with an inoculum of sewage sludge, the chemical was metabolized within 10 days and temporary formation of an intermediate was observed. The metabolite was isolated by thin-layer chromatography and determined to be 1,3-dihydro-2*H*-indol-2-one (oxindole) by UV spectroscopy (λ_{max}, 247 nm) and mass spectrometry (*m/z*, 133). The methane produced (net amount) indicated nearly complete mineralization of indole.

Aromatic *N*-heterocyclic compounds are often present in aqueous effluents associated with coal mining and processing operations (4). The environmental fate of these chemicals is of great concern because they are toxic (12) and may contaminate both surface water and groundwater.

Previous investigations of microbial metabolism of aromatic chemicals under aerobic (8) and anaerobic (13) conditions suggest that microorganisms may play a key role in determining the fate of this class of compounds. Although anaerobic metabolism by pure bacterial cultures of *N*-heterocyclic aromatic compounds has been examined (3, 5, 9, 10), detailed knowledge of transformation of these compounds by methanogenic consortia is scarce. Balba and Evans (1) indentified several intermediates and proposed a methanogenic fermentation pathway for the *N*-heterocyclic amino acid tryptophan. More recently, Wang et al. (11) reported that indole disappeared and methanogenesis was enhanced in bottles containing microbial cells bound to activated carbon to which indole was added.

The present study was designed to further explore the methanogenic fermentation of indole, as an example of aromatic *N*-heterocyclic compounds. For this purpose, we investigated the biotransformation of indole by a microbial consortium present in anaerobically digested sludge.

Culture conditions. A mineral salts medium was prepared (with 1 ml of 0.1% resazurin solution added) and handled as described by Boyd et al. (2). After being autoclaved for 15 min to remove O_2, the medium was maintained under a positive pressure of N_2 gas which was previously passed through copper filings at 300°C to remove traces of O_2. To facilitate its dissolution, crystalline indole was added to the medium when the medium temperature had cooled to 50°C; the final indole concentration was 50 μg (0.43 μmol)/ml. When the medium reached room temperature, 1.2 mg of Na_2HCO_3 and 0.12 mg of $Na_2S \cdot 9H_2O$ per ml were added and the pH was adjusted to 7.0 with a solution of HCl.

Municipal (State College, Pa.) sewage sludge was collected from primary anaerobic digesters, filtered through cheesecloth, and mixed at a ratio of 1:11 with the medium. While the inoculated medium was vigorously stirred, 100-ml portions were dispensed to N_2-flushed serum bottles (160 ml), which were subsequently closed with butyl rubber stoppers and aluminum crimp seals. Control bottles were sterilized by being autoclaved on three successive days. All bottles were incubated stationary in the dark at 35°C, and all samples (sterile, nonsterile, and with and without added indole) were prepared in duplicate. Additional bottles were frozen after various time intervals for subsequent chemical analysis.

Transfer of radioactivity from indole to oxindole was measured in 10 ml of medium in 20-ml serum bottles under conditions identical to those described above. [2-^{14}C]indole (52 mCi/mmol, 99% purity; Research Products International Corp., Mount Prospect, Ill.) was dissolved in methanol, 0.018 μCi was added per ml of medium.

Analyses. Production of methane in the serum bottles was determined by injecting 100 μl of headspace gas into a series 1800 Varian Aerograph gas chromatograph. The gas chromatograph was equipped with a thermal conductivity detector (operated at 200°C) and a Porapak Q column (600 cm, 50/80-mesh; kept at 50°C). Helium was the carrier gas at a flow rate of 40 ml/min.

Medium (2 ml) was withdrawn periodically from serum bottles, frozen immediately, and stored in a freezer. Subsequently, samples were thawed, mixed with methanol (1:1), and centrifuged (8,000 × *g*). The supernatant was then filtered through a 0.2-μm-pore nylon filter and injected onto a high-performance liquid chromatography (HPLC) system (Waters Associates, Inc., Milford, Mass.), which consisted of a model 6000A pump, a no. U6K injector, a no. 720 systems controller, a no. 480 Lamda Max variable-wavelength spectrophotometer, and a no. 730 data module. Compounds were separated by using a Radial-Pak cartridge (Nova C18, 5 μm; Waters) with a Waters radial compression module (no. RCM-100). The mobile phase consisted of water and methanol (1:1) at a flow rate of 1.5 ml/min. Quantification of indole and its transformation product (oxindole) was carried out by the external standards method at wavelengths of 271 and 247 nm, respectively. Calibration curves for

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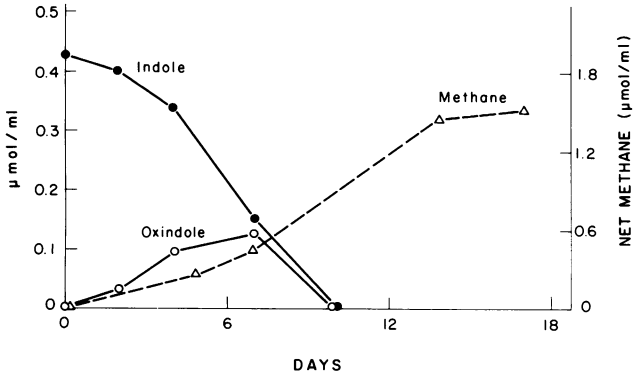

FIG. 1. Transformation of indole in a 9% solution of anaerobically digested sludge.

indole and oxindole were linear in the concentration range of 3 to 50 μg/ml (0.026 to 0.43 μmol/ml). Extraction efficiencies for indole and oxindole were 78 and 86%, respectively. All reported concentrations were multiplied by the inverse of the appropriate extraction efficiency.

Frozen serum bottles were thawed, filtered (no. 4 filter; Whatman, Inc., Clifton, N.J.), and twice extracted with 25 ml of methylene chloride. The combined methylene chloride extracts were evaporated to dryness in a flash evaporator under vacuum. The residue was dissolved in methanol and transferred to a small glass vial. The methanol was then evaporated to dryness under N_2 and dissolved in 0.01 ml of methylene chloride. Components in the solution were then separated by thin-layer chromatography on 0.25-mm-thick precoated silica gel plates (no. 60 F-254; E. Merck AG, Darmstadt, Federal Republic of Germany) in a solvent mixture of hexane-methylene chloride-ethyl acetate (6:1:3, vol/vol). The transformation product was isolated after it was extracted from the thin-layer chromatographic plate with ethyl acetate, dehydrated with sodium sulfate, and evaporated to dryness under N_2.

UV spectra of the intermediate product and indole were obtained with a spectrophotometer (model 2000; Bausch & Lomb, Inc., Rochester, N.Y.). Mass spectral data were acquired with the direct insertion probe of an AEI MS-902 mass spectrometer at an electron energy of 70 eV.

Medium to which radioactive indole was added was mixed with methanol (3:2, vol/vol) and filtered, and 100-μl injections were analyzed by HPLC. The eluent was collected at 0.5-min intervals for the entire duration of the 7.0-min chromatograms with a FOXY fraction collector (Isco, Inc., Lincoln, Nebr.). Indole and oxindole were eluted during the 4.5- to 5.5-min and 3.0- to 4.0-min intervals, respectively. Radioactivity was measured with a Beta Trac 6895 liquid scintillation counter (Tracor Analytic, Elk Grove Village, Ill.) and ScintiVerse II scintillation cocktail (Fisher Scientific Co., Fair Lawn, N.J.).

Indole metabolism. The disappearance of indole in 9% anaerobic municipal sludge was monitored by HPLC. The complete disappearance of indole required 10 days (Fig. 1). During this period, the concentration of indole in the sterile bottles remained unchanged. As the indole concentration declined, the formation of an intermediate metabolite was observed. Subsequently, this intermediate was isolated by thin-layer chromatography and identified as oxindole (1,3-dihydro-2*H*-indol-2-one). Mass-spectral analysis (*m/z*, 133; Fig. 2), UV absorbance (λ_{max}, 247 nm), and HPLC retention

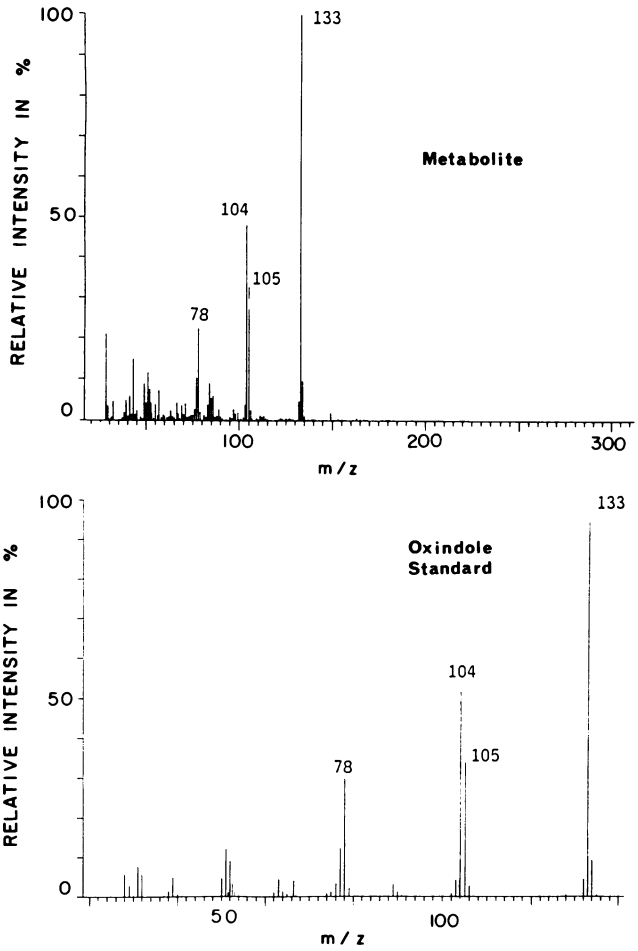

FIG. 2. Mass spectra of an oxindole standard and the intermediate of indole degradation.

time of the intermediate were identical to the same analyses performed with an authentic oxindole standard.

Additional evidence for the conversion of indole to oxindole was obtained in experiments with radioactive indole (Table 1). During the 52-day incubation period, the amount of radioactivity initially added as indole declined from 100 to 7.2%. Simultaneously, the radioactivity in the oxindole fraction rose from 0 to 42%. In a sterile control, the initial ^{14}C in the indole fraction remained constant throughout the incubation. Measurement of the concentrations of both substrates by HPLC corroborated the determinations

TABLE 1. Conversion of indole to oxindole during methanogenic metabolism in 9% sewage sludge

Day	% ^{14}C as:		% Substrate[a] as:	
	Indole	Oxindole	Indole	Oxindole
0	100	0	100	0
6	62	Tr	60	0
16	45	4.8	46	Tr
29	13	3.8	14	Tr
38	10	16	11	20
52	7.2	42	Tr	36

[a] Determined by HPLC.

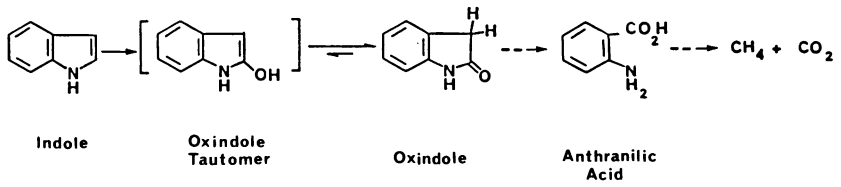

FIG. 3. Suggested pathway of methanogenic indole fermentation.

done with radioactivity. Data shown in Table 1 imply an extremely slow rate of indole transformation compared with the rate shown in Fig. 1. This retardation of indole metabolism resulted from the methanol (final medium concentration, 1%), which was the solvent for the radioactive indole. The same retardation was observed in media to which methanol was added without [^{14}C]indole.

The oxindole concentration reached a maximum at approximately day 7 and then declined below detection levels by day 10 (Fig. 1). Throughout the incubation period, however, net methane production increased, indicating that indole was mineralized. This observation was ascertained by comparing methane production in bottles containing added indole with those containing no added indole. After 17 days of incubation, the amount of methane produced corresponded to 80% of that predicted by the following stoichiometric equation: $C_8H_7N + 7H_2O \rightarrow 4.5CH_4 + 3.5CO_2 + NH_3$.

The data demonstrate that indole is metabolized via oxindole to methane by anaerobic sewage microorganisms. (Fig. 1; Table 1). Our results and those of Balba and Evans (1), who reported the isolation of indole-3-yl-acetate, indole, anthranilate, salicylate, benzoate, methane, and carbon dioxide during methanogenic tryptophan metabolism, provide a basis for suggesting a pathway for methanogenic indole fermentation (Fig. 3). The structure of oxindole implies that the initial step in methanogenic indole metabolism is hydroxylation. We show the hydroxy tautomer parenthetically in Fig. 3, but the predominant tautomeric structure after hydroxylation is the keto species, oxindole. Metabolic steps yet to be discovered presumably convert oxindole to anthranilic acid, which is degraded to methane and carbon dioxide.

Hydroxylation of an *N*-heterocyclic compound (as an initial metabolic product) is not a unique finding. Oxindole has been detected in culture filtrates of *Pseudomonas putida* during aerobic metabolism of indole (6). Stadtman et al. (10) have shown that a hydroxylation reaction occurs during the anaerobic metabolism of nicotinic acid by a *Clostridium* species; 6-oxonicotinic acid was identified as the initial fermentation product. These results indicate that both the π-electron-deficient (e.g., the pyridine nucleus of nicotinic acid) and the π-electron-excessive (e.g., the indole nucleus) aromatic compounds undergo a similar hydroxylation reaction.

The sequence of enzymatic steps which lead to ring cleavage appears to depend upon the type of aromatic ring being metabolized. Benzenoid compounds are first hydrogenated (reduced) and then undergo hydroxylation-hydrolytic ring cleavage (7). In contrast, the initial hydroxylation of nicotinate, as discussed above, is followed by reduction-hydrolytic cleavage (10). After hydroxylation, it is probable that indole undergoes a hydrolytic ring cleavage.

In summary, indole was degraded by an anaerobic microbial consortium present in digested municipal sludge. A hydroxylated intermediate was isolated and identified as oxindole; it was presumed to be the initial intermediary metabolite.

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